Dade Behring Marburg GmbH **2002/B001 - Ma 1248**
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Carbohydrate deficient transferrin (CDT)-specific antibodies, their preparation and use

The present invention relates to antibodies which, in aqueous solution, selectively bind to a transferrin-homologous carbohydrate deficient transferrin (CDT) without the latter needing to be bound to a solid phase. CDT is characterized by at least one of the two oligosaccharide chains which are normally bound to Asn 413 and/or Asn 611 of transferrin being entirely or substantially entirely lacking.

Alcoholism is a problem around the world. A number of diagnostic tests for diagnosing alcoholism have been developed in the past. Most of these tests are, however, not specific for the disorder. The test which has been developed furthest to date was introduced by Makhlouf et al. in EP-0 605 627. The antibodies disclosed therein react specifically with CDT, which was found in alcoholics but not in non-alcoholics. This made it possible to design an immunoassay with whose aid it is possible to detect CDT in alcoholics' sera. However, the disadvantage of this test is that the antigen to be detected must firstly be coupled to a solid phase, because the antibodies disclosed in EP-0 605 627 do not bind, or bind only inadequately, to CDT which is present in solution.

The object therefore was to improve the CDT detection in such a way that direct detection of CDT present in solution in a sample becomes possible and thus there is no longer a need to couple the antigen to be detected to a solid phase.

This object has surprisingly been achieved by providing antibodies which bind selectively to CDT in aqueous

solution without the latter needing to be bound to a solid phase. It has been found with the aid of epitope-mapping experiments that antibodies of the invention, in contrast to prior art antibodies, bind simultaneously to different segments of the CDT sequence. It was inferred from this that the epitopes recognized by the antibodies of the invention are discontinuous epitopes.

The present invention thus relates to an antibody which selectively binds to CDT in aqueous solution without the latter needing to be bound to a solid phase. It has been found that this antibody does not bind or binds insubstantially to the peptides P1 or P2 prepared according to EP-0 605 627, it being immaterial whether the peptides are bound to a solid phase or present in solution.

Selective binding means for the purposes of the present invention a sufficiently specific or substantially specific binding which makes it possible clearly to distinguish between CDT on the one hand and human transferrin on the other.

The term "solid phase" encompasses for the purposes of the present invention an article which consists of porous and/or nonporous, usually water-insoluble material and may have a wide variety of shapes, such as, for example, vessel, tube, microtiter plate, sphere, microparticle, rod, strip, filter paper or chromatography paper, etc. The surface of the solid phase is usually hydrophilic or can be made hydrophilic. The solid phase can consist of a wide variety of materials such as, for example, of inorganic and/or organic materials, of synthetic, of naturally occurring and/or of modified naturally occurring materials. Examples of solid phase materials are polymers such as, for example, cellulose, nitrocellulose, cellulose acetate, polyvinyl chloride,

polyacrylamide, crosslinked dextran molecules, agarose, polystyrene, polyethylene, polypropylene, polymethacrylate or nylon; ceramics; glass; metals, in particular noble metals such as gold or silver; magnetite; mixtures or combinations thereof; etc. It is also intended that the term "solid phase" include cells, liposomes or phospholipid vesicles.

The solid phase may have a coating of one or more layers, for example of proteins, carbohydrates, lipophilic substances, biopolymers, organic polymers or mixtures thereof, in order for example to diminish or to prevent nonspecific binding of constituents of samples to the solid phase or in order for example to achieve improvements in relation to the suspension stability of particulate solid phases, the storage stability, the dimensional stability or the resistance to UV light, microbes or other damaging agents.

The present invention additionally relates to an antibody which binds selectively to CDT, where the binding takes place in the region of the following segments (1) to (4) of the CDT sequence:

(1) VVARSMGGKEDLIWELL and
(2) TTEDSIAKIMNGEADAMSLDGGF and
(3) SKLSMGSGLNLSEPN and
(4) YEKYLGEEYVKAV.

The present invention further relates to an antibody of this type whose binding takes place only in the region of only three or of only two of the aforementioned segments (1) to (4) of the sequence.

In a preferred embodiment, the antibodies of the invention are monoclonal antibodies.

Very particularly preferred monoclonal antibodies are those produced by cell cultures which were deposited under the Budapest Treaty at the DSMZ Deutsche Sammlung

von Mikroorganismen und Zellkulturen GmbH, Mascheroder Weg 1b, 38124 Brunswick, Germany on April 16, 2002 (accession date at the depository) as follows:

Cell culture 01-102/01 accession number: DSM ACC2541
Cell culture 98-84/011 accession number: DSM ACC2540

Antigen-binding fragments, for example Fab, Fab', Fv or $F(ab')_2$ fragments, which can be prepared from the aforementioned antibodies of the invention by processes known to every skilled worker, are also according to the invention.

The term "antibody" generally means for the purposes of this invention not only complete antibodies but expressly also antibody fragments such as the Fab, Fv, $F(ab')_2$ or Fab' fragments already mentioned, and also chimeric, humanized, bi- or oligospecific, or single-chain antibodies; additionally aggregates, polymers and conjugates of immunoglobulins and/or fragments thereof, as long as the binding properties to the antigen or hapten are retained. Antibody fragments can be prepared for example by enzymatic cleavage of antibodies with enzymes such as pepsin or papain. Antibody aggregates, polymers and conjugates can be generated by diverse methods, e.g. by thermal treatment, reaction with substances such as glutaraldehyde, reaction with immunoglobulin-binding molecules, biotinylation of antibodies and subsequent reaction with streptavidin or avidin, etc.

An antibody can be for the purposes of this invention a monoclonal or a polyclonal antibody. The antibody can have been prepared by conventional processes, e.g. by immunization of a human or of an animal such as for example, mouse, rat, guinea-pig, rabbit, horse, sheep, goat, chicken (see also Messerschmid (1996) BIOforum, 11:500-502), and subsequent obtaining of the antiserum; or by establishment of hybridoma cells and subsequent

purification of the secreted antibodies; or by cloning and expression of the nucleotide sequences, or modified versions thereof, which encode the amino acid sequences which are responsible for the binding of the natural antibody to the antigen and/or hapten.

The present invention additionally relates to a process for preparing an antibody of the invention by immunizing a suitable experimental animal with unglycosylated transferrin or CDT, subsequently fusing the spleen cells of this experimental animal to myeloma cells, resulting in antibody-producing hybrid cells, and subsequently cloning the hybrid cells and selecting a hybrid cell clone which produces an antibody which selectively binds to CDT in aqueous solution without the latter needing to be bound to a solid phase. Finally, antibodies are obtained by a process known to the skilled worker from the hybrid cell clone selected in this way.

The present invention further relates to a process for preparing the antibody by immunizing a suitable experimental animal with unglycosylated transferrin or CDT, subsequently fusing the spleen cells of this experimental animal to myeloma cells, resulting in antibody-producing hybrid cells, and subsequently cloning the hybrid cells and selecting a hybrid cell clone which produces an antibody whose binding according to the results of an epitope mapping takes place in the region of the following segments (1) to (4) of a CDT sequence:

(1) VVARSMGGKEDLIWELL and
(2) TTEDSIAKIMNGEADAMSLDGGF and
(3) SKLSMGSGLNLSEPN and
(4) YEKYLGEEYVKAV;

followed finally by the obtaining of antibodies by a process known to the skilled worker from the hybrid cell clone selected in this way.

In place of unglycosylated transferrin or CDT, it is possible to use for the immunization of a suitable experimental animal in accordance with one of the aforementioned processes also a peptide comprising one or more of segments (1) to (4) of the sequence. The skilled worker is additionally aware that a short peptide which consists for example only of a single one or more than one of the aforementioned segments of the sequence can where appropriate be bound to a suitable carrier molecule to achieve adequate immunogenicity. Carrier molecules suitable for this purpose, for example peptides or proteins, are known to the skilled worker.

The preparation processes described above encompass the hybridoma technology which is known to every skilled worker for the preparation of monoclonal antibodies, as was published for the first time in 1975 by Köhler and Milstein and has since been modified or improved by numerous authors. Although this technology has frequently been used for preparing monoclonal antibodies from mouse cells, there are also publications which describe the preparation of monoclonal antibodies of another origin. In addition, processes for preparing antibody constructs have also been disclosed, for example humanized or bi- or oligospecific or chimeric antibodies, which can of course likewise be employed for preparing antibodies of the invention.

The present invention also relates to an immunoassay for detecting CDT in a sample; this entails an antibody of the invention described above or a corresponding antibody fragment being brought into contact with the sample and then the formation of an immune complex involving CDT being determined qualitatively or quantitatively.

Test kits for carrying out an aforementioned immunoassay, comprising an antibody of the invention or an antibody fragment of the invention are likewise an aspect of the present invention.

The present invention is additionally explained by the following examples. These serve exclusively to illustrate by way of example individual aspects of the present invention and are by no means to be understood as a restriction.

Examples

Example 1: Preparation of anti-human transferrin-Sepharose

For the affinity purification of transferrin from human sera (normal sera and alcoholics' sera), an affinity support was prepared by coupling 120 mg of anti-human transferrin (Dade Behring Marburg GmbH, Marburg, Germany) to 0.8 g of CNBr-activated Sepharose CL-4B.
120 mg of anti-human transferrin are dialyzed against 0.1M $NaHCO_3$ solution. 0.8 g of Sepharose CL-4B (Amersham Biosciences Europe GmbH, Freiburg, Germany) is washed with 0.1M $NaHCO_3$ solution and, while cooling, 1.28 g of cyanogen bromide dissolved in 5 ml of acetonitrile are added. The suspension is stirred at pH 11 and 4°C for 15 minutes. The suspension is then thoroughly washed with 0.1M $NaHCO_3$ solution. The activated Sepharose is suspended in 0.1M $NaHCO_3$ solution, and the prepared antibody solution is added and incubated at room temperature for 6 hours. The anti-human transferrin-Sepharose prepared in this way is washed with phosphate-buffered saline of pH 7.2 and stored in phosphate-buffered saline of pH 7.2 + 1 g/l NaN_3 until used.

Example 2: Isolation of human transferrin from human serum (normal serum and alcoholic's serum)

For the affinity purification of transferrin from human serum, the anti-human transferrin-Sepharose prepared in example 1 is packed into a glass column and washed with 100 ml of phosphate-buffered saline of pH 7.2 + 1g/l NaN_3. 10 ml of human serum (normal serum and alcoholic's serum) are loaded onto the column at a flow rate of 0.5 ml/minute, and the unbound proteins are removed by washing the column with 50 ml of phosphate-buffered saline of pH 7.2 + 1g/l NaN_3, 50 ml of 1M NaCl solution and 50 ml of water. The bound transferrin is eluted with 50 ml of 0.5M glycine solution whose pH has been adjusted to pH 2.5 with hydrochloric acid, immediately neutralized by adding solid tris(hydroxymethyl)aminomethane and dialyzed against phosphate-buffered saline of pH 7.2 + 1g/l NaN_3.

Example 3: Unglycosylated human transferrin

a) Recombinant unglycosylated human transferrin

Recombinant unglycosylated transferrin is prepared with the aid of conventional methods of genetic manipulation and molecular biology and is described in Mason et al. (1993) Biochemistry, 32: 5472-5479.

b) Enzymatic deglycosylation of human transferrin

60 mg of human transferrin (e.g. from Calbiochem-Novabiochem GmbH, Bad Soden, Germany) are dissolved in 8 ml of phosphate-buffered saline of pH 7.2 with 10 mM EDTA and 1 g/l (w/v) sodium decyl sulfate (from Fluka, order No.: 71443). The transferrin solution prepared in this way is heated to 37°C in a water bath, and 180 units (3 units/mg transferrin) of N glycosidase F (from Roche, order No. 1365193) are added. The mixture is incubated in a water bath at 37°C for 17 hours. The completeness of deglycosylation is investigated by SDS-PAGE (Duan et al. (1998) Applied Biochemistry and Biotechnology, 69: 217-224).

Example 4: Preparation of monoclonal antibodies according to the prior art

The preparation of monoclonal antibodies according to the prior art took place as described in the patent EP-0 605 627 B1 by immunization with transferrin-specific peptide sequences P1 and P2. The following hybrids/monoclonal antibodies were obtained:

Antibody number:	Specificity:
01-32/062	anti-P1
00-177/012	anti-P1
00-187/016	anti-P2
00-187/027	anti-P2

Example 5: Preparation of the monoclonal antibodies of the invention

a) Immunization of mice

BALB/c mice were each immunized intraperitoneally with 20 µg of unglycosylated transferrin in complete Freund's adjuvant. A booster was given after 4 weeks with in each case 20 µg of unglycosylated transferrin in incomplete Freund's adjuvant (from ICN Biomedical GmbH, Eschwege, Germany) and after 8 weeks with in each case 20 µg of unglycosylated transferrin without Freund's adjuvant. For the last 3 days before the fusion, the mice were given intravenous boosters each of 20 µg of unglycosyated transferrin.

b) Fusion

After the mice had been sacrificed by CO_2 inhalation, the spleens were removed and single-cell suspensions in serum-free Dulbecco's modified Eagle Medium (DMEM, from CC Pro GmbH, Neustadt/W, Germany) were prepared. The cells were centrifuged (652 g) and washed 2× in DMEM. The cell count was then determined by Trypan Blue staining. 2×10^7 myeloma cells (Sp2/0) were added to

about 10^8 spleen cells. After centrifugation (360 g), the supernatant was discarded, 1 ml of polyethylene glycol solution (PEG 400, from Merck Eurolab, Bruchsal, Germany; about 50% strength in DMEM) was added to the cell pellet and incubated after resuspension at 37°C for 1 minute. About 10 ml of DMEM were then added dropwise, and the mixture was incubated at room temperature for 2 to 4 minutes. The fused cells were spun down (326 g) and the pellet was resuspended in DMEM + 20% FCS (fetal calf serum, from Biowhittaker Europe, Verviers, Belgium) + HAT solution (from CC Pro GmbH, Neudstadt/W, Germany) and introduced into 24-well cell culture plates (from Costar). The approximate cell concentration per well was 5×10^4 to 5×10^6 cells.

2-3 weeks later, the resulting cell colonies (hybrids) were removed and transferred into new culture plates.

c) Determination of the antibody specificity

The specificity of the antibodies released into the cell culture was tested in a first test step using immunizing antigen-coated microtiter plates (from Nunc, type B), coating 1 µg/ml ≈ 0.015 µg/well.

100 µl of cell culture supernatant (dilution 1:2) were pipetted into each well of the microtiter plate and incubated at +15 to +25°C for 1 hour. After the plate had been washed twice with washing solution POD (OSEW; from Dade Behring, Marburg, Germany), 100 µl of anti-mouse IgG/F(ab')$_2$-POD conjugate (from Dade Behring, Marburg, Germany) were introduced into each well and then incubated at +15 to +25°C for 1 hour. After the plate had been washed a further two times, 100 µl of chromogen TMB solution (from Dade Behring, Marburg, Germany) were introduced into each well and incubated at +15 to +25°C for a further 30 minutes. After the incubation, 100 µl of stop solution POD (from Dade Behring, Marburg, Germany) were introduced into each

well, and the microtiter plate was evaluated in a BEP II (Behring ELISA processor II, from Dade Behring, Marburg, Germany) at 450 nm.

In a second test step, the hybrids were checked as described above using microtiter plates (from Nunc, type B), which were coated with human transferrin (for example from Calbiochem-Novabiochem GmbH, Bad Soden, Germany). Coating 1 µg/ml ≈ 0.015 µg/well.

The results are listed in table 1.

Table 1: Determination of the antibody specificity by evaluation of the microtiter plates in a BEP II (Behring ELISA processor II) at 450 nm

	Extinction at 450 nm	
Hybrid number	Unglycosylated human transferrin	Human transferrin
98-22/026 (569)	> 2.5	negative
98-23/07 (45)	> 2.5	negative
98-22/0104 (572)	1.739	negative
98-84/011 (1)	> 2.5	negative
01-102/01 (113)	> 2.5	negative

Key: negative = extinction$_{(450\ nm)}$ < 0.1 OD; no gradation of the signal on dilution of the hybrids investigated

d) Cloning

Single cells of hybrids which produce the antibodies of the invention (binding to unglycosylated human transferrin but not to human transferrin) were cloned using a micromanipulator (from Leitz, Wetzlar, Germany). The clones 98-84/011 and 01-102/01 obtained in this way were deposited on April 16, 2002 at the DSMZ Deutsche Sammlung Mikroorganismen und Zellkulturen GmbH, Mascheroder Weg 1b, Brunswick, Germany, under

accession number DSM ACC2540 (98-84/011) and DSM ACC2541 (01-102/01).

e) Determination of the antibody subclass

The subclass of the antibodies 98-84/011 and 01-102/01 was determined using IsoStrip™ mouse monoclonal antibody isotyping kit from Boehringer Mannheim, Germany, to be IgG_1 for 98-84/011 and 01-102/01.

f) Antibody production

To produce larger quantities of antibodies, the appropriate cell clones are transferred into roller bottles (from Corning Costar Deutschland, Bodenheim) and expanded at +37°C to the desired final volume. A 0.22 µm filtration of the roller culture suspension is then carried out to remove the cells. The antibody solution, which is now cell-free, is concentrated in an ultrafilter (separation limit 30 000 dalton) and then purified.

g) Antibody purification

The resulting antibody solution is rebuffered to 0.14M phosphate buffer of pH 8.6, and loaded onto a chromatography column packed with rProtein A Sepharose Fast Flow (from Amersham Pharmacia) (1 ml of rProtein A Sepharose Fast Flow is employed per 10 mg of antibodies to be purified). All unbound components are removed by washing the column with 0.14M phosphate buffer of pH 8.6. The bound antibody is eluted from the column with 0.1M citric acid of pH 3.0 and dialyzed against 0.05M sodium acetate + 0.5M NaCl + 0.05M Tris + 0.01% sodium azide of pH 7.0.

Example 6: Determination of the specificity of the antibodies for <u>solid phase-bound</u> antigens: comparison of antibodies of the invention with *prior art* antibodies

The specificity of the antibodies obtained was tested using a) microtiter plates (Nunc, type B) coated with unglycosylated transferrin, coating 1 µg/ml ≈ 0.015 µg/well, b) microtiter plates (Nunc type B) coated with human transferrin, coating 1 µg/ml ≈ 0.015 µg/ well, c) microtiter plates (Nunc, type B) coated with peptide P1, coating 3 µg/ml ≈ 0.045 µg/well and d) microtiter plates (Nunc type B) coated with peptide P2, coating 3 µg/ml ≈ 0.045 µg/well.

100 µl of monoclonal antibodies (1 µg/ml) were pipetted into each well of the microtiter plate and incubated at +15 to +25°C for 1 hour. After the plate had been washed twice with washing solution POD (OSEW; from Dade Behring, Marburg, Germany), 100 µl of anti-mouse IgG/F(ab')$_2$-POD conjugate (from Dade Behring, Marburg, Germany) were introduced into each well and then incubated at +15 to +25°C for 1 hour. After the plate had been washed a further two times, 100 µl of chromogen TMB solution (from Dade Behring, Marburg, Germany) were introduced into each well and incubated at +15 to +25°C for a further 30 minutes. After the incubation, 100 µl of stop solution POD (from Dade Behring, Marburg, Germany) were introduced into each well, and the microtiter plate was evaluated in a BEP II (from Dade Behring, Marburg, Germany) at 450 nm.

The results are listed in table 2.

Table 2: Determination of the antibody specificity by evaluation of microtiter plates in BEP II at 450 nm.

		Extinction at 450 nm			
Antibody		Un-glycosylated human transferrin	Human transferrin	Peptide P1	Peptide P2
Antibodies of the invention	98-22/026	1.578	negative	negative	negative
	98-23/07	2.497	negative	negative	negative
	98-22/0104	1.179	negative	negative	negative
	98-84/011	> 2.5	negative	negative	negative
	01-102/01	2.432	negative	negative	negative
Prior art anti-peptide P1 antibodies	00-177/012	1.063	0.157	> 2.5	negative
	01-32/062	> 2.5	0.151	> 2.5	negative
Prior art anti-peptide P2 antibodies	00-187/016	2.339	negative	negative	> 2.5
	00-187/027	> 2.5	negative	negative	> 2.5

Key: negative = $extinction_{450\ nm}$ < 0.1 OD; no gradation of the signal on dilution of the hybrids investigated

The antibodies of the invention show only a reaction with unglycosylated transferrin, while the prior art antibodies show a reaction with each peptide and with the unglycosylated transferrin bound to the solid phase.

Example 7: Determination of the specificity of the antibodies for antigens in solution: comparison of antibodies of the invention with prior art antibodies

a) Microtiter plates (Nunc, type B) were coated with the monoclonal antibodies of the invention and with prior art monoclonal antibodies. Coating concentration 1 µg/ml ≈ 0.015 µg/well.

100 µl of a geometric dilution series starting at 200 µg/ml of a) human transferrin, b) enzymatically deglycosylated human transferrin, c) human transferrin from normal serum and d) human transferrin from alcoholic's serum were pipetted into the wells of the microtiter plate and incubated at +15 to +25°C for 1 hour. After the plate had been washed twice with washing solution POD (OSEW; from Dade Behring, Marburg, Germany), 100 µl of anti-human transferrin-POD conjugate (from Dade Behring, Marburg, Germany) were introduced into each well and then incubated at +15 to +25°C for 1 hour. After the plate had been washed a further two times, 100 µl of chromogen TMB solution (from Dade Behring, Marburg, Germany) were introduced into each well and incubated at +15 to +25°C for a further 30 minutes. After the incubation, 100 µl of stop solution POD (from Dade Behring, Marburg, Germany) were introduced into each well, and the microtiter plate was evaluated in a BEP II (from Dade Behring, Marburg, Germany) at 450 nm.

The results are listed in table 3.1 and 3.2.

Table 3.1: Determination of the reactivity by evaluation of microtiter plates in a BEP II at 450 nm.

		Extinction at 450 nm													
		Antibodies of the invention			Prior art antibodies					Antibodies of the invention			Prior art antibodies		
Antigen	Conc. [µg/ml]	98-23/07	98-84/011	01-102/01	01-32/062	00-187/016	00-187/027	Antigen	Conc. [µg/ml]	98-23/07	98-84/011	01-102/01	01-32/062	00-187/016	00-187/027
Human transferrin	200	1.790	2.5	0.137	negative	0.508	0.553	Unglycosylated human transferrin	200	2.500	2.500	1.773	0.388	2.500	2.500
	100	0.664	2.5	negative	negative	0.230	0.291		100	2.500	2.500	1.582	0.262	2.193	2.500
	50	0.541	2.5	negative	negative	0.123	0.170		50	2.500	2.500	1.570	0.160	1.406	2.133
	25	0.491	2.5	negative	negative	negative	negative		25	2.500	2.500	1.601	0.104	0.714	1.134
	12.5	0.320	2.5	negative	negative	negative	negative		12.5	2.500	2.500	1.274	negative	0.442	0.588
	6.25	0.158	2.5	negative	negative	negative	negative		6.25	2.500	2.500	1.238	negative	0.233	0.320
	3.125	negative	1.880	negative	negative	negative	negative		3.125	2.500	2.500	1.230	negative	0.133	0.183
	1.56	negative	0.604	negative	negative	negative	negative		1.56	2.500	2.500	0.880	negative	negative	negative
	0.781	negative	0.407	negative	negative	negative	negative		0.781	2.500	2.500	0.890	negative	negative	negative
	0.391	negative	0.284	negative	negative	negative	negative		0.391	2.500	2.500	0.722	negative	negative	negative
	0.195	negative	0.169	negative	negative	negative	negative		0.195	2.500	2.500	0.436	negative	negative	negative

negative: $\text{extinction}_{(450\ nm)} < 0.1$ OD

positive: $\text{extinction}_{(450\ nm)} \geq 0.1$ OD

Table 3.2: Determination of the reactivity by evaluation of microtiter plates in a BEP II at 450 nm.

		Extinction at 450 nm													
		Antibodies of the invention			Prior art antibodies					Antibodies of the invention			Prior art antibodies		
Antigen	Conc. [µg/ml]	98-23/07	98-84/011	01-102/01	01-32/062	00-187/016	00-187/027	Antigen	Conc. [µg/ml]	98-23/07	98-84/011	01-102/01	01-32/062	00-187/016	00-187/027
Human	200	1.309	2.5	0.188	negative	0.142	0.192	Human	200	0.508	2.5	negative	negative	0.118	0.133
trans-	100	0.229	2.5	0.116	negative	negative	0.158	trans-	100	0.660	2.5	negative	negative	negative	negative
ferrin	50	0.177	2.5	negative	negative	negative	0.111	ferrin	50	0.306	2.5	negative	negative	negative	negative
from	25	0.141	2.5	negative	negative	negative	negative	from	25	0.252	2.5	negative	negative	negative	negative
normal	12.5	0.100	2.5	negative	negative	negative	negative	alco-	12.5	0.181	2.5	negative	negative	negative	negative
serum	6.25	negative	2.5	negative	negative	negative	negative	holic's	6.25	0.101	2.5	negative	negative	negative	negative
	3.125	negative	2.5	negative	negative	negative	negative	serum	3.125	negative	2.5	negative	negative	negative	negative
	1.56	negative	1.234	negative	negative	negative	negative		1.56	negative	2.5	negative	negative	negative	negative
	0.781	negative	0.745	negative	negative	negative	negative		0.781	negative	2.5	negative	negative	negative	negative
	0.391	negative	0.450	negative	negative	negative	negative		0.391	negative	1.676	negative	negative	negative	negative
	0.195	negative	0.245	negative	negative	negative	negative		0.195	negative	0.920	negative	negative	negative	negative

negative: $\text{extinction}_{(450\ nm)} < 0.1$ OD

positive: $\text{extinction}_{(450\ nm)} \geq 0.1$ OD

b) Microtiter plates (Nunc, type B) were coated with the monoclonal antibodies of the invention and with prior art monoclonal antibodies. Coating concentration 3 µg/ml ≈ 0.045 µg/well.

100 µl of a geometric dilution series starting at a 1:10 dilution of a) normal serum and b) alcoholic's serum were pipetted into the wells of the microtiter plate and incubated at +15 to +25°C for 1 hour. After the plate had been washed twice with washing solution POD (OSEW; from Dade Behring, Marburg, Germany), 100 µl of anti-human transferrin-POD conjugate (from Dade Behring, Marburg, Germany) were introduced into each well and then incubated at +15 to +25°C for 1 hour. After the plate had been washed a further two times, 100 µl of chromogen TMB solution (from Dade Behring, Marburg, Germany) were introduced into each well and incubated at +15 to +25°C for a further 30 minutes. After the incubation, 100 µl of stop solution POD (from Dade Behring, Marburg, Germany) were introduced into each well, and the microtiter plate was evaluated in a BEP II (from Dade Behring, Marburg, Germany) at 450 nm.

The results are listed in table 4.

Table 4: Determination of the reactivity by evaluation of microtiter plates in a BEP II at 450 nm.

		Extinction at 450 nm													
		Antibodies of the invention				Prior art antibodies				Antibodies of the invention				Prior art antibodies	
Antigen	Dilution	98-23/07	98-22/0104	98-84/011	01-102/01	01-32/062	00-187/016	Antigen	Dilution	98-23/07	98-22/0104	98-84/011	01/102/01	01-32/062	00-187/016
Normal human serum	1:10	0.318	0.512	2.5	0.150	negative	negative	Alcoholic human serum	1:10	0.603	0.861	2.5	0.220	negative	negative
	1:20	0.212	0.313	2.5	negative	negative	negative		1:20	0.367	0.545	2.5	0.148	negative	negative
	1:40	0.146	0.193	2.5	negative	negative	negative		1:40	0.259	0.391	2.5	0.103	negative	negative
	1:80	0.107	0.104	2.5	negative	negative	negative		1:80	0.165	0.205	2.5	negative	negative	negative
	1:160	negative	negative	2.5	negative	negative	negative		1:160	0.128	0.155	2.5	negative	negative	negative
	1:320	negative	negative	1.605	negative	negative	negative		1:320	0.110	0.118	2.5	negative	negative	negative
	1:640	negative	negative	0.936	negative	negative	negative		1:640	negative	negative	2.5	negative	negative	negative

negative: $\text{extinction}_{(450\ nm)} < 0.1$ OD

positive: $\text{extinction}_{(450\ nm)} \geq 0.1$ OD

The antibodies of the invention make it possible to differentiate clearly between transferrin (in normal serum) and CDT (in alcoholic's serum), while the prior art antibodies show no reaction with both sera.

Example 8: Epitope mapping

Scans of overlapping peptides derived from the sequence of human transferrin (13-mer peptides, 11 amino acids overlapping) were prepared using the SPOT synthesis technology. The methods are described in: Wenschuh, H. et al. (2000) Coherent membrane supports for parallel microsynthesis and screening of bioactive peptides, *Biopolymers (Peptide Science)*, 55:188-206. The peptides were coupled at the C terminus to a cellulose support and carry a reactivity tag at the N terminus. After the peptides had been cleaved off cut-out SPOTs (96-well microtiter plate), they were coupled to activated glass chips. The incubation protocol for these glass chips is as follows:

Prior art monoclonal antibodies

- equilibration in TBS buffer, pH 8.0
- blocking buffer, pH 8.0, 2 h
- antibody incubation (3 µg/ml in blocking buffer, pH 8.0), 2 h
- washing with TBS (0.05% Tween20)
- incubation with anti-mouse IgG-POD in blocking buffer, pH 8.0, 2 h
- washing with TBS (0.05% Tween20) 3 × 5 min
- chemoluminescence detection (Lumi-Imager, Roche Diagnostics)

Antibody 98-84/011 of the invention

- equilibration in TBS buffer, pH 8.0
- blocking buffer, pH 8.0, 2 h
- antibody incubation (3 µg/ml) in blocking buffer, pH 8.0, 2 h
- washing with TBS (0.05% Tween20) 3 × 5 min
- chemoluminescence detection (Lumi-Imager, Roche Diagnostics)

The antibody of the invention was directly labeled with peroxidase. The method is described in the literature: Wilson, M.B. and Nakane, P.K. (1978) Recent

developments in the periodate method of conjugating horseradish peroxidase (HRPO) to antibodies, In: Immunofluorescence and Related Staining Techniques (Eds.: Knapp, W.; Holubar, K.; Wick, G.) pp. 215-224.

After evaluation of the investigation, the binding peptides for the prior art antibodies are revealed to be as follows:

Prior art antibodies against peptide 1

1. VLAENY**NKSDNCE**
2. AENY**NKSDNCE**DT
3. NY**NKSDNCE**DTPE
4. **NKSDNCE**DTPEAG

Prior art antibodies against peptide 2

1. VHKILRQQ**QHLFG**
2. KILRQQ**QHLFG**SN
3. LRQQ**QHLFG**SNVT
4. QQ**QHLFG**SNVTDC
5. **QHLFG**SNVTDCSG

The recognized sequences are identical to the peptides employed for the immunization.

The antibody 98-84/011 of the invention reacts with four dominant segments of the sequence:

1. VVAR**SMGGKEDLI**
2. AR**SMGGKEDLI**WE
3. **SMGGKEDLI**WELL

4. TTEDSIAKIM**NGE**
5. SIAKIM**NGE**ADAM
6. AKIM**NGE**ADAMSL
7. IM**NGE**ADAMSLDG
8. **NGE**ADAMSLDGGF

9. SK**LSMGSGLNLSE**

10. **LSMGSGLNLSE**PN

11. YEKYLGEEYVKAV

Region 1.-3. is located in the N-terminal domain of transferrin, while regions 4.-8., 9.-10. and 11. are located in the C-terminal domain and represent a discontinuous epitope.